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Role of Peptide Hydrophobicity in the Mechanism of Action of α-Helical Antimicrobial Peptides▽

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In the present study, the 26-residue amphipathic α-helical antimicrobial peptide V13K$_L$ (Y. Chen et al., J. Biol. Chem. 2005, 280:12316–12329, 2005) was used as the framework to study the effects of peptide hydrophobicity on the mechanism of action of antimicrobial peptides. Hydrophobicity was systematically decreased or increased by replacing leucine residues with less hydrophobic alanine residues or replacing alanine residues with more hydrophobic leucine residues on the nonpolar face of the helix, respectively. Hydrophobicity of the nonpolar face of the amphipathic helix was demonstrated to correlate with peptide helicity (measured by circular dichroism spectroscopy) and self-associating ability (measured by reversed-phase high-performance liquid chromatography temperature profiling) in aqueous environments. Higher hydrophobicity was correlated with stronger hemolytic activity. In contrast, there was an optimum hydrophobicity window in which high antimicrobial activity could be obtained. Decreased or increased hydrophobicity beyond this window dramatically decreased antimicrobial activity. The decreased antimicrobial activity at high peptide hydrophobicity can be explained by the strong peptide self-association which prevents the peptide from passing through the cell wall in prokaryotic cells, whereas increased peptide self-association had no effect on peptide access to eukaryotic membranes.

Antibiotic resistance, due to the extensive clinical use of classical antibiotics (22, 32), has become a great concern in recent years, prompting an urgent need for a new class of antibiotics. Antimicrobial peptides have been proposed as potent candidates of a new class of antibiotics, with characteristics including an ability to kill target cells rapidly, an unusually broad spectrum of activity, activity against some of the more serious antibiotic-resistant pathogens in clinics, and the relative difficulty in selecting resistant mutants in vitro (13, 35). Although the exact mode of action of antimicrobial peptides has not been established, it is generally accepted that the cytoplasmic membrane is the main target of antimicrobial peptides, whereby peptide accumulation in the membrane causes increased permeability and a loss of barrier function, resulting in the leakage of cytoplasmic components and cell death (13, 28).

Factors believed to be important for antimicrobial activity have been identified, including peptide hydrophobicity, the presence of positively charged residues, an amphipathic nature that segregates basic and hydrophobic residues, and secondary structure. Recently, Hodges and coworkers increased this list to include (i) the importance of a lack of structure in benign medium (nondenaturing conditions; see Materials and Methods) but an inducible structure in the presence of the hydrophobic environment of the membrane, (ii) the presence of a positively charged residue in the center of the nonpolar face of amphipathic cyclic β-sheet and α-helical peptides as a determinant for locating the peptides at the interface region of prokaryotic membranes and decreasing transmembrane penetration into eukaryotic membranes, and (iii) the importance of peptide self-association in an aqueous environment to the biological activities of these peptides (5, 18). Many studies have previously shown that peptide self-association in the membrane-bound state correlated with antimicrobial activity (30) but that peptide self-association in an aqueous environment had no effect on antimicrobial activity. Hydrophobicity and amphipathicity are considered crucial parameters for the peptides whose sole target is the cytoplasmic membrane (5, 11).

Recently, we designed an antimicrobial peptide, V13K$_L$, with a high level of activity against various gram-negative and gram-positive bacteria and, more importantly, negligible hemolytic activity (5, 8). In the present study, in order to investigate the role of hydrophobicity in the mechanism of action of α-helical antimicrobial peptides, we systematically decreased or increased the hydrophobicity of peptide V13K$_L$ on the nonpolar face and report here that hydrophobicity has dramatically different effects on the biological activities in prokaryotic and eukaryotic cells.

MATERIALS AND METHODS

Peptide synthesis and purification. Synthesis of the peptides was carried out by solid-phase peptide synthesis by using *t*-butyloxycarbonyl chemistry and 4-methylbenzhydrylamine resin (0.97 mmol/g) and purification by reversed-phase high-performance liquid chromatography (RP-HPLC), as described previously (6, 7). The purity of the peptides was verified by analytical RP-HPLC and was further characterized by mass spectrometry and amino acid analysis.

Analytical RP-HPLC and temperature profiling of peptides to calculate the peptide self-association parameter (P_A). The RP-HPLC peptide retention times (t_Rs) were determined at temperatures from 5°C to 80°C in 3°C increments, as described previously (18, 20).

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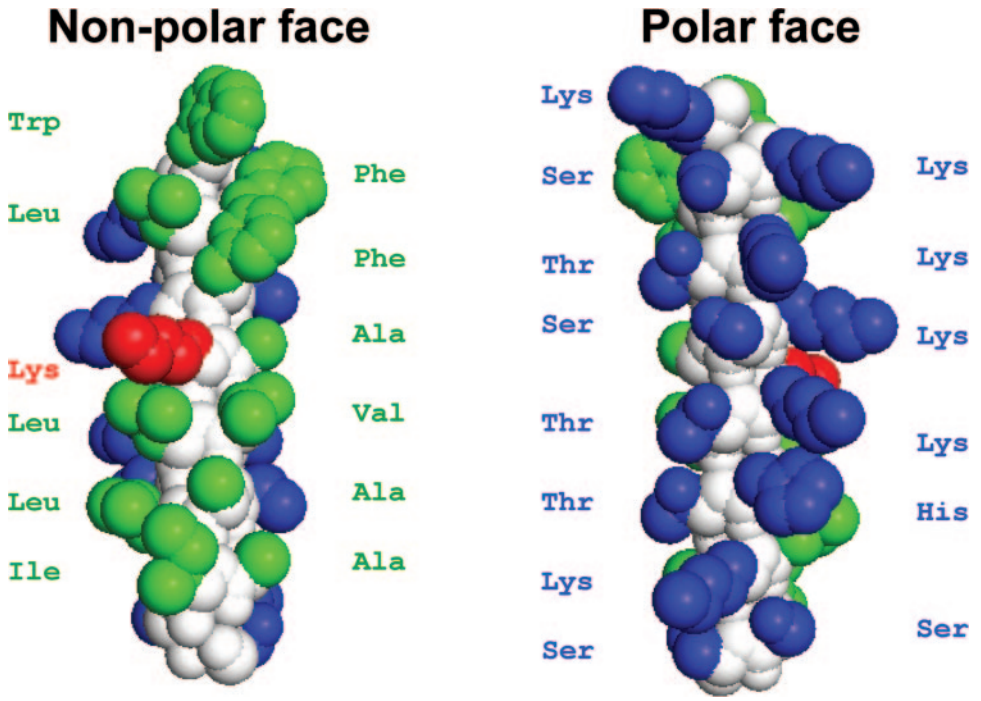

FIG. 1. Space-filling model of parent peptide $V13K_L$. Hydrophobic amino acids on the nonpolar face of the helix are colored green; hydrophilic amino acids on the polar face of the helix are colored blue; the peptide backbone is colored white. The Lys substitution at position 13 ($V13K_L$) on the nonpolar face of the helix is colored red. The models were created with the PyMOL (version 0.98) program. The peptide sequences are shown in Table 1.

Characterization of helical structure. The mean residue molar ellipticities of the peptides were determined by circular dichroism (CD) spectroscopy with a J-720 spectropolarimeter (Jasco, Easton, MD) at 5°C under benign (nondenaturing) conditions (50 mM KH_2PO_4, K_2HPO_4, 100 mM KCl, pH 7.4), hereafter referred to as KP buffer, as well as in the presence of an α-helix inducing solvent, 2,2,2-trifluoroethanol (TFE; 50 mM KH_2PO_4, K_2HPO_4, 100 mM KCl, pH 7.4), with the buffer and TFE present at 1:1 (vol/vol). A 10-fold dilution of an ~500 μM stock solution of the peptide analogs was loaded into a 1-mm fused silica cell, and its ellipticity was scanned from 190 to 250 nm.

***Pseudomonas aeruginosa* strains used in this study.** Strain PAO1 was isolated from a human wound in 1955 in Australia (14); strain WR5 was isolated from a burn patient at Walter Reed Army Hospital, Washington, DC, in 1976 and is a natural *toxA* mutant isolate but is virulent in experimental mouse models (3, 24); strain PAK was originally isolated at Memorial University, St. John's, Newfoundland, Canada, and is widely used in the analysis of pili (12, 33); strain PA14 was originally isolated as a clinical isolate in 1995 at the Massachusetts General Hospital, Boston, and is virulent in a variety of plant and animal models of infection (26); strain M2 was originally isolated in 1975 from the gastrointestinal tract of a healthy CF1 mouse, University of Cincinnati College of Medicine, and Shriners Burns Institute, Cincinnati, OH, and is virulent in a burn mouse model of *P. aeruginosa* infection (29); and strain CP204 was isolated from a cystic fibrosis patient in 1989 at the National Jewish Medical and Research Center, Denver, CO. All strains have been maintained at −80°C in the laboratory of Michael Vasil.

Measurement of antimicrobial activity (MICs). MICs were determined by a standard microtiter dilution method in brain heart infusion (BHI) medium. Briefly, cells were grown overnight at 37°C in BHI broth and were diluted in the same medium. Serial dilutions of the peptides were added to the microtiter plates in a volume of 50 μl, followed by the addition of 50 μl of bacteria to give a final inoculum of 5×10^5 CFU/ml. The plates were incubated at 37°C for 24 h, and the MICs were determined as the lowest peptide concentration that inhibited growth.

Measurement of hemolytic activity (minimal hemolytic concentration [MHC]). Peptide samples were added to 1% human erythrocytes in phosphate-buffered saline (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4) and the reaction mixtures were incubated at 37°C for 18 h in microtiter plates. Twofold serial dilutions of the peptide samples were carried out in order to determine the concentration that produced no hemolysis. This determination was made by withdrawing aliquots from the hemolysis assays and removing unlysed erythrocytes by centrifugation ($800 \times g$). Hemoglobin release was determined spectrophotometrically at 570 nm. The hemolytic activity was determined as the maximal peptide concentration that caused no hemolysis of erythrocytes after 18 h. The control for no release of hemoglobin was a sample of 1% erythrocytes without any peptide added. Since erythrocytes were in an isotonic medium, no detectable release (<1% of that released upon complete hemolysis) of hemoglobin was observed from this control during the course of the assay.

RESULTS

Peptide design. Peptide $V13K_L$ is a 26-residue amphipathic peptide which adopts an α-helical conformation in a hydrophobic environment and which contains a hydrophilic lysine residue in the center of the nonpolar face (position 13) (Fig. 1 and Fig. 2) (5, 8). In the present study, we used peptide $V13K_L$ as a framework to systematically alter peptide hydrophobicity on the nonpolar face of the helix by replacing alanine residues with the more hydrophobic leucine residues to increase hydrophobicity or by changing leucine residues to alanine residues to decrease hydrophobicity. The peptide sequences are shown in Table 1. Figure 2 shows the peptide analogs represented as helical nets. Since there are three alanine residues on the nonpolar face of peptide $V13K_L$, three single Leu-substituted analogs were synthesized with an Ala→Leu substitution at positions 12 (A12L), 20 (A20L), and 23 (A23L); two double Leu-substituted peptides (A12L/A20L and A12L/A23L) were also made to increase peptide hydrophobicity further, and peptide A12L/A20L/A23L exhibited the highest hydrophobicity, with all three alanine residues on the nonpolar face substituted by leucine residues. In contrast, in order to decrease the hy-

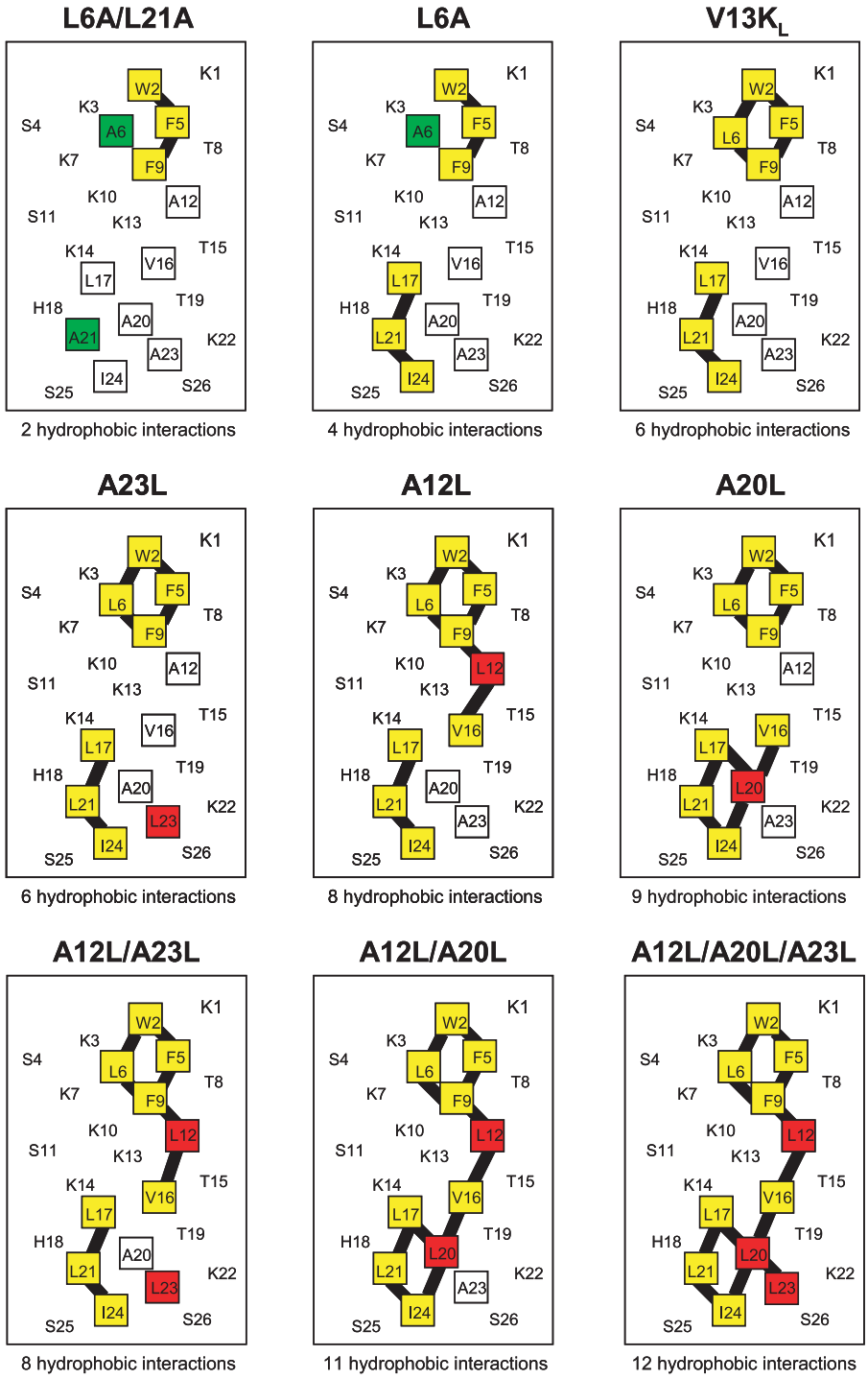

FIG. 2. Helical net representation of the sequences of parent peptide V13K$_L$ and analogs (with different leucine substitutions). The hydrophobic amino acid residues on the nonpolar faces are boxed. The substituting alanine residues are colored green, the substituting leucine residues are colored red, and the amino acid residues involved in the $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions are colored yellow and red. The $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions are shown as black bars, and the numbers of hydrophobic interactions on the nonpolar face are indicated. The one-letter code is used for the amino acid residues. The white boxes indicate hydrophobes not involved in $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions.

drophobicity of peptide V13K$_L$, leucine at position 6 was selected to make a Leu→Ala (L6A) substitution due to its participation in the $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions on the nonpolar face (a peptide sequence in an α-helical conformation allows a side chain in position i to interact with a side chain in position $i + 3$ or $i + 4$ along the sequence). One double-Ala-substituted peptide (L6A/L21A) was also made to decrease the peptide hydrophobicity further.

TABLE 1. Sequences of peptides used in this study

Denotation	Peptide sequences[a]
L6A/L21A	Ac-KWKSF*A*KTFKSAKKTVLHTA*A*KAISS-amide
L6A	Ac-KWKSF*A*KTFKSAKKTVLHTALKAISS-amide
$V13K_L$	Ac-KWKSFLKTFKSAKKTVLHTALKAISS-amide
A23L	Ac-KWKSFLKTFKSAKKTVLHTALK***L***ISS-amide
A12L	Ac-KWKSFLKTFKS***L***KKTVLHTALKAISS-amide
A20L	Ac-KWKSFLKTFKSAKKTVLHT***L***LKAISS-amide
A12L/A23L	Ac-KWKSFLKTFKS***L***KKTVLHTALK***L***ISS-amide
A12L/A20L	Ac-KWKSFLKTFKS***L***KKTVLHT***L***LKAISS-amide
A12L/A20L/A23L	Ac-KWKSFLKTFKS***L***KKTVLHT***L***LK***L***ISS-amide
C[b]	Ac-ELEKGGLEGEKGGKELEK-amide

[a] Peptide sequences are shown by using the one-letter code for amino acid residues; Ac, N^{α}-acetyl; amide, C-terminal amide; in the sequences, the boldface and italic letters denote the substituting amino acids at the nonpolar face of $V13K_L$ (see Fig. 2 for details).

[b] Peptide C is a random coil peptide used as a control in the RP-HPLC temperature profiling experiment.

We have shown that these interactions among large hydrophobes like leucine residues stabilize the α-helical structure in α-helical peptides. It is clear that, for single Leu-substituted analogs, the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions in the peptides is in the order of A20L > A12L > A23L (9, 8, and 6 hydrophobic interactions, respectively); for peptides with double leucine substitutions, A12L/A20L has more $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions than A12L/A23L (11 versus 8 hydrophobic interactions); peptide A12L/A20L/A23L exhibits the highest number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions (12 hydrophobic interactions) among all the peptide analogs. In addition, the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions in the Ala-substituted peptides is smaller than that in peptide $V13K_L$ and in the order of L6A/L21A < L6A (two and four hydrophobic interactions, respectively).

Peptide hydrophobicity. RP-HPLC retention behavior is a particularly good method to represent apparent peptide hydrophobicity, and the retention times of peptides are highly sensitive to the conformational status of peptides upon interaction with the hydrophobic environment of the column matrix (5, 6). The nonpolar face of an amphipathic α-helical peptide represents a preferred domain for binding to the hydrophobic matrix of a reversed-phase column (37). In this study, the observed hydrophobicity of the peptides (as expressed by RP-HPLC retention time) is in the order L6A/L21A < L6A < $V13K_L$ < A23L < A12L < A20L < A12L/A23L < A12L/A20L < A12L/A20L/A23L (t_R range, 65.6 to 100.4 min; Table 2). For the three single Leu-substituted peptides, the leucine substitution at position 20 was more effective at increasing the hydrophobicity of $V13K_L$ than the Leu substitution at position 12, which increased the hydrophobicity of $V13K_L$ more than a Leu substitution at position 23 did. As expected, with the combined effects of leucine substitutions at positions 12 and 20, peptide A12L/A20L exhibited greater hydrophobicity than peptide A12L/A23L. Triple-Leu-substituted peptide A12L/A20L/A23L showed the highest hydrophobicity among the peptide analogs (t_R, 100.4 min; Table 2).

From Table 2 and Fig. 2, the hydrophobicity of $V13K_L$ was altered in two ways: first, by replacing alanine with leucine or leucine with alanine, the intrinsic hydrophobicity of the side chain was changed (15); and second, peptide hydrophobicity is influenced by the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions, which affects the continuity of the hydrophobic surface on the nonpolar face of the peptide. For example, peptides A23L, A12L, and A20L share the same intrinsic hydrophobicity; however, due to the different number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions by positional differences of the substituting leucines in the nonpolar face of $V13K_L$, the three analogs exhibited dramatically different hydrophobicities according to their RP-HPLC retention times (Table 2; Fig. 2). From Table 2, the number of the $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions on the nonpolar face of the peptide analogs correlates with the observed hydrophobicities of the peptides with the same amino acid composition, i.e., the single- and double-substituted analogs.

Secondary structures of peptides. Figure 3 shows the CD spectra of the peptide analogs in different environments, i.e., under benign conditions (in KP buffer) (Fig. 3A) and in KP buffer with 50% TFE to mimic the hydrophobic environment of the membrane (Fig. 3B). In Fig. 3A and Table 2, peptides L6A/L21A, L6A, $V13K_L$, A23L, and A12L showed negligible

TABLE 2. Biophysical data of $V13K_L$ peptide analogs

Peptide[a]		Hydrophobicity[b]		Benign[c]		50% TFE[c]		P_A[e]
No.	Denotation	t_R	$\Delta t_R(X - V13K_L)$	$[\theta]_{222}$	% Helix[d]	$[\theta]_{222}$	% Helix[d]	
1	L6A/L21A	65.6	−9.2	−850	3	−18,900	56	1.0
2	L6A	70.8	−4.0	−1,250	4	−22,900	68	1.4
3	$V13K_L$	74.8	0	−1,400	4	−27,000	80	2.1
4	A23L	82.6	7.8	−3,900	12	−27,550	82	4.5
5	A12L	83.9	9.1	−5,700	17	−28,800	86	5.5
6	A20L	85.5	10.7	−10,350	31	−30,150	90	5.4
7	A12L/A23L	91.4	16.6	−12,200	36	−33,000	98	7.0
8	A12L/A20L	92.8	18.0	−13,400	40	−33,200	99	9.3
9	A12L/A20L/A23L	100.4	25.6	−16,550	49	−33,550	100	11.3

[a] Peptide sequences are shown in Table 1.

[b] Peptides are ordered by increasing t_R by RP-HPLC at pH 2 and room temperature, which is a measure of overall hydrophobicity. $\Delta t_R(X - V13K_L)$, difference in retention times between peptide analogs and the native peptide, $V13K_L$, as a measure of the change in hydrophobicity.

[c] The mean $[\theta]_{222}$ values (in deg · cm^2 · $dmol^{-1}$) at wavelength 222 nm were measured at 5°C under benign conditions (100 mM KCl, 50 mM PO_4, pH 7.4) or in benign buffer containing 50% TFE by circular dichroism spectroscopy.

[d] The helical content (in percent) of a peptide relative to the molar ellipticity value of peptide A12L/A20L/A23L in the presence of 50% TFE.

[e] P_A, dimerization parameter of each peptide during the RP-HPLC temperature profiling, which is the maximal retention time difference of ($t_R{}^t - t_R{}^5$ for peptide analogs) − ($t_R{}^t - t_R{}^5$ for control peptide C) within the temperature range; $t_R{}^t - t_R{}^5$ is the retention time difference of a peptide at a specific temperature ($t_R{}^t$) compared with that at 5°C ($t_R{}^5$).

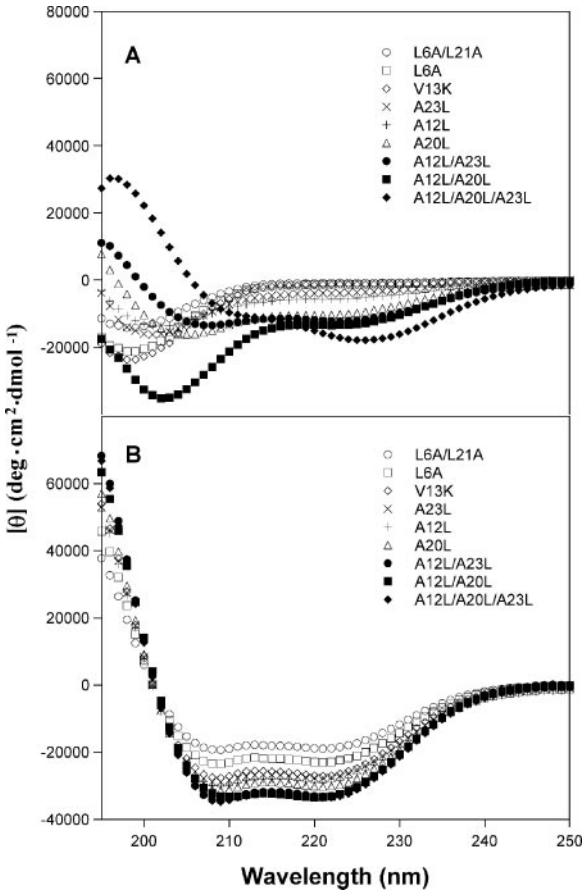

FIG. 3. CD spectra of peptides $V13K_L$ and its analogs in buffer (50 mM KH_2PO_4, K_2HPO_4, 100 mM KCl, pH 7.4) (A) and in the presence of buffer-TFE (1:1 [vol/vol]) (B) at pH 7.4 and 5°C.

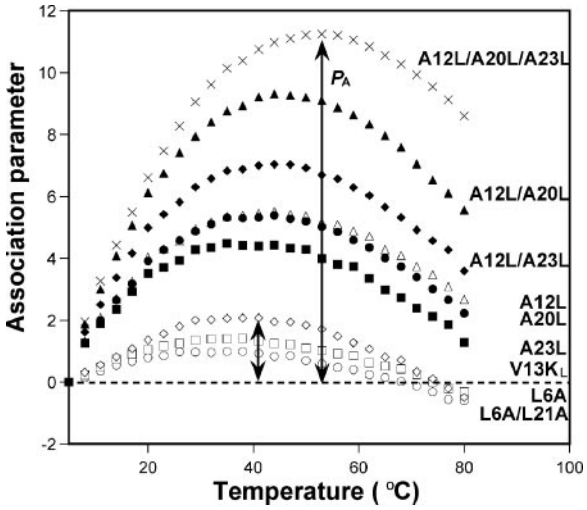

FIG. 4. Peptide self-association ability as monitored by RP-HPLC with increasing temperature. Symbols: open circles, L6A/L21A; open squares, L6A; open diamonds, $V13K_L$; closed squares, A23L; closed circles, A20L; open triangles, A12L; closed diamonds, A12L/A23L; closed triangles, A12L/A20L; and X, A12L/A20L/A23L; double-headed arrows, P_A values for A12L/A20L/A23L and $V13K_L$ of 11.3 and 2.1, respectively (Table 2).

helical structures in KP buffer; A20L and the two peptides with double Leu substitutions exhibited different degrees of helical structure; peptide A12L/A20L/A23L exhibited a unique spectrum under benign conditions compared to the spectra of the other analogs, in which the ratio of $[\theta]_{222}/[\theta]_{208}$ (where $[\theta]_{222}$ and $[\theta]_{208}$ are the residue molar ellipticities at 222 and 208 nm, respectively) is more than 1, which has been used as a criterion of stable fully folded dimers in two-stranded α-helical coiled coils (17). Regardless of the different secondary structures of the peptides in KP buffer, a highly helical structure may be induced by the nonpolar environment of 50% TFE, a mimic of hydrophobicity, and the α-helix-inducing ability of the membrane (Fig. 3B and Table 2). It is worthy of note that even though peptides L6A/L21A and L6A were in a strong helix-inducing environment of 50% TFE, they were not fully folded helical structures. The helicities of the peptides in benign buffer and in 50% TFE relative to that of peptide A12L/A20L/A23L in 50% TFE were determined (Table 2). It is clear that both under benign conditions and in the presence of 50% TFE, the relative helicities of the peptides are in the order L6A/L21A < L6A < $V13K_L$ < A23L < A12L < A20L < A12L/A23L < A12L/A20L < A12L/A20L/A23L. It is clear that increasing hydrophobicity is also correlated with the increasing α-helical structure of the peptides in aqueous environments ($R = 0.948$) (see Fig. 5B and Table 2). Alteration of the peptide hydrophobicity by replacing alanine residues with leucine residues or leucine residues with alanine residues changed the peptide helicity in benign KP buffer. In addition, although alanine has the highest helical propensity (38), these results show that the hydrophobicity of the nonpolar face of an amphipathic molecule also plays an important role in stabilizing the peptide secondary structure.

Peptide self-association. Studies have shown that peptide self-association (i.e., the ability to dimerize) in aqueous solutions is a very important parameter in obtaining an understanding of antimicrobial activity (2, 5, 18). If the self-association ability of a peptide is too strong in aqueous medium, it could decrease the ability of the peptide to dissociate, pass through the capsule and cell wall of microorganisms, and penetrate into the cytoplasmic membrane to kill target cells. The capsule is composed primarily of high-molecular-weight polysaccharides. The cell wall of gram-negative bacteria includes the outer membrane, which contains proteins, lipopolysaccharides, porins, and lipids of the bilayer, as well as the peptidoglycan layer between the outer and inner (cytoplasmic) membranes. Which components of the cell wall restrict the access of the folded peptide dimers to the cytoplasmic membrane remain unknown.

The ability of peptides to self-associate was determined by the technique of RP-HPLC temperature profiling (5, 18, 20). Figure 4 shows the retention behaviors of the peptides, after normalization to the retention behavior of control peptide C to deduct the general temperature effects (5, 18, 20). Control peptide C is a monomeric random coil peptide in both aqueous and hydrophobic media; thus, its retention behavior within the temperature range of 5°C to 80°C represents only the general effects of temperature on peptide retention behavior, i.e., a linear decrease in the peptide retention time with increasing

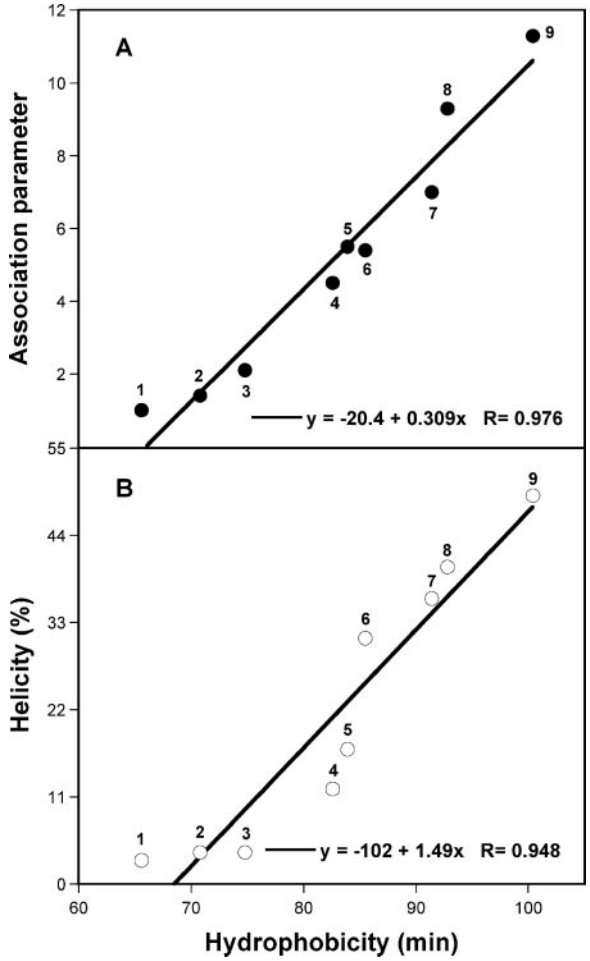

FIG. 5. Relationships of peptide hydrophobicity and association ability (A) and helicity (B). Hydrophobicity is expressed as the retention times of peptides in RP-HPLC at room temperature (Table 2). Solid symbols, self-association parameter (A); open symbols, helicity (B). The peptides denoted by the numbers are shown in Table 2.

temperature due to greater solute diffusivity and enhanced mass transfer between the stationary and mobile phases at higher temperatures. As shown in Fig. 4, P_A (the maximum change in peptide retention time relative to that of random coil peptide C) increases with increasing the hydrophobicities of the peptides. In Table 2, the order of the peptide self-association ability of the peptide analogs is similar to the order of peptide hydrophobicity, although no significant difference in P_A values was observed between A12L and A20L. By simply replacing three alanine residues with three leucine residues, we increased the self-association ability of $V13K_L$ by more than fivefold (from 2.1 for $V13K_L$ to 11.3 for A12L/A20L/A23L). In contrast, by replacing leucine residues with alanine residues, we decreased the peptide hydrophobicity, the continuity of the nonpolar face, and peptide helicity in an aqueous environment, resulting in the decrease of peptide self-association ability (from 2.1 for $V13K_L$ to 1.4 for L6A and 1.0 for L6A/L21A). Increasing temperature not only disrupts dimerization/oligomerization but at some point can cause unfolding of the α-helical structure, resulting in the loss of the nonpolar face of the amphipathic α-helical peptides (i.e., the preferred binding domain in RP-HPLC) and, hence, reduced retention times as the peptides become increasingly random coils (20).

Figure 5A shows the relationships between peptide hydrophobicity and self-association ability and helicity in an aqueous environment. Figure 5A demonstrates that there is a linear correlation ($R = 0.976$) between peptide self-association and the hydrophobicity of the nonpolar face of the peptide, i.e., peptides with higher hydrophobicities on their nonpolar face generally showed stronger self-associating ability in solution, since dimers can be formed by interactions between the nonpolar faces of two molecules (Fig. 5A and Table 2).

Antimicrobial activities. The antimicrobial activities of the peptides, as shown in Table 3, were determined with a diverse group of *P. aeruginosa* clinical isolates. *Pseudomonas* is a genus of gram-negative bacteria with high intrinsic resistance to traditional antibiotics. Resistance levels have been increasing further in recent years, and *P. aeruginosa* is also known to produce

TABLE 3. Biological activities of $V13K_L$ analogs

Peptide		Hemolytic activity		Antimicrobial activity							
No.	Denotation	MHC (μg/ml)	Fold[a]	MIC (μg/ml)[b]							Fold[d]
				PAO1	WR5	PAK	PA14	M2	CP204	GM[c]	
1	L6A/L21A	1,000	0.3	500	>500	>500	>500	>500	>500	—[e]	<0.1
2	L6A	1,000	0.3	125	250	250	500	250	125	223	0.2
3	$V13K_L$	250	1.0	31.3	250	125	62.5	31.3	7.8	49.6	1.0
4	A23L	62.5	4.0	31.3	62.5	62.5	31.3	31.3	15.6	35.1	1.4
5	A12L	31.3	8.0	15.6	62.5	31.3	31.3	15.6	15.6	24.8	2.0
6	A20L	31.3	8.0	7.8	31.3	31.3	15.6	15.6	7.8	15.6	3.2
7	A12L/A23L	15.6	16.0	15.6	250	15.6	62.5	31.3	7.8	31.2	1.6
8	A12L/A20L	8.0	32.0	62.5	125	62.5	125	62.5	62.5	78.7	0.6
9	A12L/A20L/A23L	4.0	62.5	500	500	500	500	500	500	500	0.1

[a] The fold increase in hemolytic activity compared to that for the parent peptide, $V13K_L$. Hemolytic activity (MHC, which is the maximal peptide concentration showing no hemolysis after 18 h) was determined with human red blood cells.

[b] MIC for six *P. aeruginosa* clinical isolates. The relative susceptibilities of these *P. aeruginosa* strains to ciprofloxacin, with the number 1 denoting the most susceptible strain, are as follows: PAO1, 4; WR5, 1; PAK, 4; PA14, 8; M2, 8; CP204, 64.

[c] GM, geometric mean of the MICs for the six *P. aeruginosa* clinical isolates.

[d] The fold improvement in antimicrobial activity (geometric mean data) compared to that of parent peptide $V13K_L$.

[e] —, for peptide L6A/L21A, no activity against WR5, PAK, PA14, M2, and CP204 was measured at a concentration of 500 μg/ml; thus, no geometric mean MIC could be calculated.

proteolytic enzymes which make it even less susceptible to antimicrobial peptides (25). The *P. aeruginosa* strains used in the present study exhibited a wide range of susceptibilities to ciprofloxacin (Table 3). The *P. aeruginosa* strain most resistant to ciprofloxacin was CP204, a clinical isolate from cystic fibrosis patients, which was, in contrast, the strain tested in this study that was the most susceptible to our antimicrobial peptides. The geometric mean MICs for six *P. aeruginosa* strains were calculated (Table 3) to provide an overall evaluation of the antimicrobial activities of the peptides with different hydrophobicities. The fold improvement was calculated by comparing the geometric mean MIC of each peptide to that of the parent peptide, $V13K_L$. Among the peptide analogs tested, peptide A20L showed the strongest activity against *P. aeruginosa* strains, with an overall geometric mean MIC of 15.6 μg/ml, which is a 3.2-fold improvement compared to that of the parent peptide, $V13K_L$. Considering that in our previous study (5), $V13K_L$ was the best lead compound among 21 peptide analogs, on the basis of therapeutic indices, against various gram-negative and gram-positive bacteria, this is a significant improvement in peptide antimicrobial activity. In contrast, peptide A12L/A20L/A23L exhibited negligible activity against all six *P. aeruginosa* strains, with MICs of 500 μg/ml; and peptide L6A/L21A showed no activity against five *P. aeruginosa* strains and had a MIC of 500 μg/ml for strain PAO1. In Table 3, the antimicrobial activity of peptide $V13K_L$ was improved with an increase in peptide hydrophobicity (from $V13K_L$ to A20L) and was weakened with a further increase in hydrophobicity (from A20L to A12L/A20L/A23L). In contrast, decreasing the hydrophobicity of $V13K_L$ weakened the antimicrobial activity to a total loss of the antimicrobial activity (from $V13K_L$ to L6A and L6A/L21A).

It is generally accepted that increasing the hydrophobicity of the nonpolar face of the amphipathic α-helical peptides would also increase the antimicrobial activity (2, 9, 34). The striking result from our antibacterial assay was that hydrophobicity has two effects on peptide antimicrobial activity: at a relative lower level of hydrophobicity, an increase in peptide hydrophobicity caused an improvement in antimicrobial activity until an optimal hydrophobicity was reached (Table 3; Fig. 6); in contrast, peptide antimicrobial activity was weakened dramatically with further increases in hydrophobicity beyond the optimum, even resulting in the dramatic loss of antimicrobial activity of peptide A12L/A20L/A23L in this study (Fig. 6; Table 3).

Hemolytic activities. The hemolytic activities of the peptides against human erythrocytes were determined as a measure of peptide toxicity toward higher eukaryotic cells (2, 31, 34). The parent peptide, $V13K_L$, exhibited no hemolysis against human red blood cells at a concentration of 250 μg/ml after 18 h of incubation at 37°C (Table 3); in addition, it showed no hemolytic activity at a concentration of 500 μg/ml after 8 h of incubation at 37°C (data not shown). It is clear that with a decrease in peptide hydrophobicity, the hemolytic activity of the peptide analogs L6A/L21A and L6A was weakened to no hemolysis at a concentration of 1,000 μg/ml; in contrast, with an increase in peptide hydrophobicity, the hemolytic activity of the peptide analogs gradually became stronger, from 62.5 μg/ml for A23L to 4.0 μg/ml for A12L/A20L/A23L, which were 4-fold to 62.5-fold increases in hemolysis compared to that of the parent peptide, $V13K_L$, respectively (Table 3).

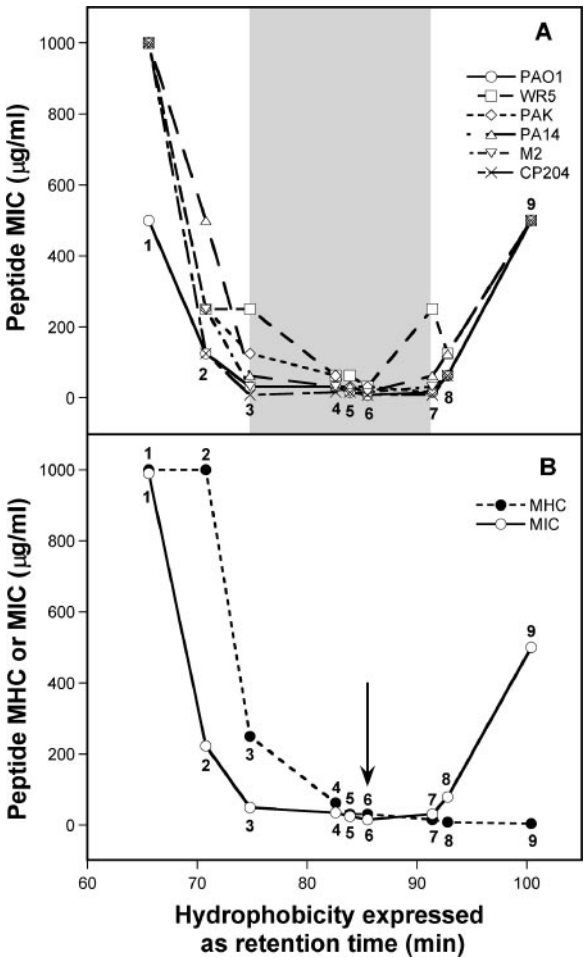

FIG. 6. Relationships of peptide hydrophobicity and antimicrobial ability (MIC) and hemolytic activity (MHC). (A) The shaded area shows the optimal hydrophobicity zone for antimicrobial activity. (B) Hydrophobicity is expressed as the retention times of peptides in RP-HPLC at room temperature. The arrow denotes the optimal antimicrobial activity, based on the geometric mean of the MICs for the six *P. aeruginosa* clinical isolates. The peptides denoted by the numbers are shown in Table 3. In the case of peptide 1 (Table 3), there was no measurable activity against five of the six *P. aeruginosa* strains and a value of 1,000 μg/ml was used for these five *P. aeruginosa* strains in panel A. A value of 1,000 μg/ml was used as the representative MIC for the six *P. aeruginosa* strains in panel B.

The influence of peptide hydrophobicity on hemolytic activity is shown in Fig. 6B. It is generally accepted that increasing the hydrophobicity of the nonpolar face of amphipathic α-helical peptides would increase the hemolytic activity. In the present study, it is clear that peptide hydrophobicity correlated with peptide hemolytic activity, in that the more hydrophobic the peptide was, the stronger the hemolytic activity against erythrocytes was, which is consistent with previous results (2, 4, 5). In addition, although the higher helicities and the stronger self-association abilities of peptides are associated with higher hydrophobicities in aqueous solution, these parameters seem to have no effect on preventing peptide molecules from entering into the membrane of human red blood cells.

DISCUSSION

In our previous study, we proposed a "membrane discrimination" mechanism of action for antimicrobial peptides whose sole target is the biomembrane (5, 8), based on a "barrel-stave" mechanism (10) in eukaryotic cells and a "carpet" mechanism (28) in prokaryotic cells. We believe that peptide specificity between eukaryotic and prokaryotic cells depends upon the compositional difference in the lipids between the two types of membranes (1). It is well known that eukaryotic cell membranes, in contrast to prokaryotic membranes, are generally characterized by zwitterionic phospholipids, a relatively large amount of cholesterol and sphingomyelin, and the absence of the high, inside negative transmembrane potential that is present in prokaryotic membranes (19, 39). Hence, if the peptides form pores/channels in the hydrophobic core of the eukaryotic bilayer, they would cause the hemolysis of erythrocytes; in contrast, for prokaryotic cells, the peptides lyse cells in a detergent-like mechanism, as described by the carpet mechanism (28). Our "membrane discrimination" mechanism is consistent with the results of previous studies of model membranes, which demonstrated that the pore formation mechanism ("barrel-stave" mechanism) was used by antimicrobial peptides in zwitterionic membranes, while a detergent-like mechanism ("carpet" mechanism) for the peptides was shown when the peptides interacted with negatively charged membranes (16, 23). Of course, model membrane studies do not address the complexity of the cell envelope and its contributions to the biological activity of these peptides.

The observation that there is a correlation between peptide hydrophobicity and hemolytic activity can be explained by the membrane discrimination mechanism. Peptides with higher hydrophobicities will penetrate deeper into the hydrophobic core of the red blood cell membrane (31), causing stronger hemolysis by forming pores or channels, which may explain the reason why A12L/A23L and A12L/A20L exhibited stronger hemolytic activities than single-Leu-substituted peptides and why A12L/A20L/A23L showed the strongest hemolytic activity in this study (Table 3; Fig. 6B). For peptides L6A/L21A and L6A, low hydrophobicity prevents peptide molecules from entering the cytoplasmic membrane of erythrocytes to cause hemolysis (Table 3; Fig. 6B).

For peptide antimicrobial activity, since the insertion of the molecules into the hydrophobic core is not necessary to lyse bacterial cells during the antibacterial action, peptides need only lie at the interface, parallel with the membrane surface, allowing their hydrophobic surface (pointing inward) to interact with the hydrophobic component of the lipid and the positively charged residues (pointing outward) to interact with the negatively charged head groups of the phospholipids. There is structural information supporting this location to the interface region in membranes (21, 27, 28). Thus, it is reasonable to assume that increasing peptide hydrophobicity to a certain extent helps peptide molecules reach the interface from an aqueous environment and improve antimicrobial activity. In this study, the improvement of antimicrobial activity from peptide $V13K_L$ to peptide A20L can represent such an advantage of increasing hydrophobicity. In contrast, further increases in hydrophobicity will cause stronger peptide dimerization in solution, which in turn results in the monomer-dimer equilibrium favoring the dimer conformation. Peptide dimers are in their folded α-helical conformation and would be inhibited from passing through the capsule and cell wall to reach the target membranes, unlike the unstructured monomer. Hence, with increasing hydrophobicity the antimicrobial activities of peptides A12L/A23L and A12L/A20L become weaker than the single-Leu-substituted analogs. For the extreme example of the triple-Leu-substituted analog, A12L/A20L/A23L, the loss of antimicrobial activity may be explained to be due to its very strong dimerization ability in aqueous environments. Hence, the peptide exists mainly as a highly positively charged and stable α-helical dimer in solution; and we speculate that it would not pass through the bacterial capsule and cell wall, whereas peptides that do not dimerize and that exist mainly as unstructured monomers containing one-half of the number of positively charged residues compared to that in the dimer would pass through the bacterial capsule and cell wall. In contrast, there is no polysaccharide-based cell wall in eukaryotic cells; thus, A12L/A20L/A23L caused severe hemolysis against human red blood cells, in which the hydrophobicity of the bilayer causes the rapid dissociation of dimers to monomers and entry into the bilayer to form channels/pores. In addition, the higher content of zwitterionic phospholipids and the large amount of cholesterol in eukaryotic cell membranes compared to the contents of bacterial membranes may also supply a more hydrophobic environment, which would promote dimer-to-monomer dissociation and enhance activity. The fact that the antimicrobial activities of peptides L6A/L21A and L6A become weaker with decreasing hydrophobicity than parent peptide $V13K_L$ can be attributed to the stronger solubility of these peptides in an aqueous environment; and even if the peptides reach the membrane surface (through an initial attraction of the positively charged residues in the peptide with the negatively charged surface of the phospholipid head groups), the overall hydrophobicity of the peptide must be large enough to allow it to partition into the hydrophobic component of the lipid, where the peptide α-helical structure is induced to maximize the hydrophobic interactions of the nonpolar face of the amphipathic α-helix and the lipid. Exactly how the peptide then disrupts the membrane is not well understood (see the work of Zhang et al. [36] for a more extensive discussion of the interaction of antimicrobial peptides with membranes). We believe that there is a threshold of hydrophobicity related to peptide antimicrobial activity; that is, one may adjust the peptide hydrophobicity to obtain the optimal antimicrobial activity, as shown by the shaded area in Fig. 6A. This is the first report clearly demonstrating that decreasing or increasing peptide hydrophobicity beyond the optimal hydrophobicity window resulted in a decrease in antimicrobial activity and that the decrease in antimicrobial activity is a result of peptide dimerization. Figure 6A also shows that this hydrophobicity window is similar for the six different strains of *P. aeruginosa* tested.

Conclusions. The increasing hydrophobicity of α-helical antimicrobial peptides resulted in stronger hemolysis in erythrocytes, while for antimicrobial activity, there is a threshold hydrophobicity at which optimal antimicrobial activity can be obtained; i.e., decreasing peptide hydrophobicity reduces antimicrobial activity; in contrast, increasing peptide hydrophobicity to a certain extent will improve antimicrobial activity and

a further increase in hydrophobicity will result in a decrease in antimicrobial activity, probably due to increased dimerization, which prevents access to the membrane in prokaryotic cells.

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This report is a tribute to and in memory of Robert Bruce Merrifield, Nobel laureate, who died in May 2006. Merrifield was the postdoctoral mentor of R.S.H. from 1971 to 1974 at Rockefeller University, New York, NY, and introduced that author to the field of peptide chemistry. One area of Merrifield's research was in the area of antimicrobial peptides.

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